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㊴ Novel DNA, cloned DNA, recombinant plasmid containing the DNA, microorganism containing the recombinant plasmid and
process for their production.

㊷ The invention relates to a DNA which codes for a
polypeptide with interferon activity, a cloned DNA showing
complementarity to human interferon mRNA, a recombinant
plasmid containing such DNA, a microorganism containig
the recombinant plasmid and a process for producing said
DNA, said cloned DNA, said recombinant plasmid and said
microorganism.

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"Novel DNA, Cloned DNA, Recombinant Plasmid Containing
the DNA, Microorganism Containing the Recombinant Plasmid
and Process for their Production"

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The present invention relates to a DNA which codes for a
polypeptide with interferon activity and to a recombinant
plasmid containing the DNA. The present invention also
pertains to a microorganism containing the recombinant
35 plasmid.

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5   Interferon is a glycoprotein (molecular weight
approx. 20,000) with antiviral activity, discovered by Isaacs
and Lindenmann in 1957. Subsequent studies have indicated
antitumor activity of the substance in addition to antiviral
activity and hence a wide clinical application of this
10 substance is expected. For instance, it has been reported
that interferon may be effectively used to treat various
viral diseases, osteosarcoma and mammary carcinoma.

       However, because of its high species-specificity,
only the interferon derived from human cells can be used for
15 human application. At present, the interferon which is being
used for administration has a relative activity of about 10^6
(International units) per 1 mg, which corresponds to a purity
of about 0.1 - 0.01%.

       Moreover, the use of the interferon is quite limited
20 because of difficulties in mass production. At present only
about 1% of the interferon requirement even for clinical
tests (10^{13} units per annum) can be met. For these reasons
there is a great need to develop technology to produce human
interferon in high purity, with ease and in large quantities.
25   To this end, a novel technique has been developed
for producing interferon with ease and in a large quantity
by inserting a human interferon gene into a plasmid DNA (for
instance plasmid DNA derived from Escherichia coli) with
30 recombinant DNA (deoxyribonucleic acid) technology.

35

In accordance with the present invention, a DNA which codes for a polypeptide with interferon activity is prepared using the human interferon messenger RNA as a template and a novel recombinant plasmid containing the DNA is prepared. In addition, the recombinant plasmid may be inserted into a host microorganism.

The DNA which codes for a polypeptide with interferon activity and the recombinant plasmid containing the DNA have been obtained for the first time by the present inventors. The DNA and the recombinant plasmid may be used, inter alia, for amplification of human interferon in bacteria such as Escherichia coli. Such bacteria are then useful for the production of human interferon in large quantities at low cost.

The DNA and the recombinant plasmid of the present invention are prepared by the following general procedure.

First, cytoplasmic RNA is extracted from (1) human fibroblast, MG63 cells or others induced by poly(I): poly(C) (a double stranded RNA composed of polyinosinic acid and polycytidylic acid) or other inducers, (2) human leucocyte, lymphoblastic cells, NAMALWA cells or others induced by Sendai virus or other inducers, or (3) lymphocytes induced by various mitogens or other inducers. From this RNA, the human interferon messenger RNA (hereinafter messenger RNA is referred to as mRNA) containing poly A (polyadenylic acid) is isolated. A double stranded DNA is synthesized, for example, by reverse transcriptase, with the mRNA preparation having high interferon mRNA activity as a template. A recombinant is obtained by inserting the synthesized DNA into a vector DNA such as Escherichia coli plasmid DNA by the technique of in vitro DNA recombination. The recombinant is labelled with a radio isotope for use as a probe. Recombinant plasmids having an inserted portion which is

complementary to the human interferon mRNA are selected.
The DNA which codes for a polypeptide with interferon
activity is recovered from the recombinant plasmid and the
base sequence of the DNA is determined.

5

Fig. 1 illustrates restriction endonuclease maps
of:

10   (a)   a gene which shows complementarity to the human
fibroblast interferon mRNA in the recombinant #319 used to
make a novel recombinant plasmid #319-13; and

        (b)   a gene which shows complementarity to the human
fibroblast interferon mRNA in the novel recombinant plasmid
15 #319-13.

20   The present invention relates to a DNA which codes
for a polypeptide with interferon activity, a recombinant
plasmid containing the DNA and a microorganism containing
the recombinant plasmid.

        The DNA of the present invention may be a cloned
25 DNA showing complementarity to the human interferon mRNA, a
cloned DNA which codes for a polypeptide with interferon
activity or a cloned DNA which codes for human interferon
polypeptide.   Especially a DNA which encompasses the entire
coding region of the human fibroblast interferon mRNA is a
30 preferred example of the DNA of the present invention.

        The recombinant plasmid of the present invention
is a recombinant plasmid wherein the DNA mentioned above is
inserted in a vector DNA such as pBR322, pCR1, pMB9 or pSC1.

        The recombinant plasmids named #319 and #319-13
35 are preferred examples of a recombinant plasmid according to
the invention.

        The DNA and the recombinant plasmid are inserted
in a host microorganism and the transformant can be used to

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produce a substance having interferon activity.

As the host microorganism, <u>Escherichia</u> <u>coli</u> X1776 is preferably used.

An example of the processes of producing the DNA, the recombinant plasmid and the transformant of the present invention is as follows.

First, human fibroblasts may be obtained from fetus-derived foreskin, or the like. A small amount of interferon is then added to a culture fluid of human fibroblasts to prime the interferon synthesis by human fibroblasts, to which poly(I): poly(C) is added to induce the synthesis of interferon mRNA. Cycloheximide is added simultaneously to increase the level of interferon mRNA. At an appropriate time (about 4 hours) after the human fibro-blasts have been superinduced in the above manner, cells are collected and destroyed and the nuclei are removed. Cytoplasmic total RNA is extracted with phenol, or the like. The RNA can also be extracted by destroying the whole cells, extracting both DNA and RNA with, for example, phenol, and degrading and removing the DNA with DNAase.

Further, interferon mRNA can also be extracted from MG63 cells induced by poly(I): poly(C) or other inducers, human leucocyte or lymphoblastic cells induced by Sendai virus or other inducers, and lymphocytes induced by various mitogens or other inducers.

The thus extracted RNA is dissolved in a salt solution of NaCl or KCl at a high concentration such as 0.5M and put on a column of oligo (dT) cellulose to adsorb mRNA having poly(A) on the column. Elution is carried out with water, a salt solution at a low concentration such as 10 mM Tris-HCl buffer, or the like to isolate mRNA having poly(A).

The isolated mRNA is fractionated by sucrose density gradient centrifugation. Interferon mRNA activity in each fraction is checked by determining interferon activity (antiviral activity) of the protein which is synthesized in oocytes of African claw toad (<u>Xenopus</u> <u>laevis</u>) after micro-injecting a part of the mRNA in each fraction. The determina-tion of interferon activity is carried out according to the

method described in Japan J. Microbiol. 18, 449-456, (1974).

Then, a DNA showing complementarity to the mRNA is synthesized in vitro by a reverse transcriptase, which is obtained from avian myeloblastosis virus, using, as the template, an mRNA having the highest interferon mRNA activity.

The synthesis is carried out as follows.

An mRNA is reacted at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 60 min.) with oligo(dT), $MgCl_2$ (e.g. 5 mM), NaCl (e.g. 30 mM), mercaptoethanol (e.g. 5 mM) and Tris-HCl buffer (e.g. pH 8.0, 40 mM) using a reverse transcriptase together with deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) (e.g. 0.5 mM each) as substrates.

The thus obtained reaction product is subjected to deproteinization with, for example, phenol, and the template RNA is removed by alkali or ribonuclease treatment. A double stranded DNA is synthesized by a reverse transcriptase in a similar way as the synthesis of the DNA showing complementarity to mRNA described above except that mRNA is replaced by DNA and oligo(dT) is omitted.

By using Escherichia coli DNA polymerase I which can be obtained from Escherichia coli MRE 600, or the like, instead of reverse transcriptase, the same double stranded DNA can be synthesized.

After the double stranded DNA which is synthesized by the above described procedure is treated with Nuclease S_1 which can be obtained from Aspergillus oryzae in the presence of $ZnCl_2$ (e.g. 1 mM), sodium acetate buffer (e.g. 0.1 M, pH 4.5), NaCl (e.g. 0.2 M), etc., deoxyadenine chains are formed at both 3' ends of the synthesized DNA by incubating with a terminal transferase purified from calf thymus in the presence of potassium cacodylate buffer (e.g. pH 7.6, 0.14 M), Tris (base) (e.g. 0.03 M), dithiothreitol (e.g. 0.1 mM), $CoCl_2$ (e.g. 1 mM) and dATP (e.g. 1 mM) at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 20 min.)

On the other hand, a plasmid DNA which is used as a vector DNA, e.g. Escherichia coli plasmid pBR322 DNA [Gene vol. 2, p. 95-113 (1977)], is cleaved at one site by treating with a restriction endonuclease EcoRI, which can be obtained, for example, from Escherichia coli RY13, in the presence of Tris HCl buffer (e.g. pH 7.5, 10 mM), $MgCl_2$ (e.g. 6 mM), NaCl (e.g. 0.1 M), mercaptoethanol (e.g. 6 mM), or the like and then treated with phage λ-derived exonuclease, which can be obtained, for example, from Escherichia coli W3102 (λ cI851) x 13), in the presence of Na-glycine buffer (e.g. pH 9.5, 0.1 M), $MgCl_2$ (e.g. 5 mM), or the like. Thereafter deoxythymidine chains are formed at both 3' ends in the same way as for the above-described synthesized double stranded DNA by using dTTP instead of dATP.

Synthetic double stranded DNA and plasmid DNA which are chain-elongated at both 3' ends as described above are incubated at an appropriate temperature for an appropriate period with Tris-HCl buffer (e.g. pH 7.5, 50 mM), NaCl (e.g. 0.1 M), EDTA (e.g. 5 mM), or the like and hybridized with hydrogen bonds formed by adenine and thymine. Then, a transformable Escherichia coli strain, e.g. Escherichia coli χ1776 (Molecular Cloning of Recombinant DNA, Scott, W. A. & Werner, R. edited, Academic Press p. 99-114, 1977) is transformed with the hybridized DNA by the method of Enea et al. (J. Mol. Biol. vol. 96, p. 495-509, 1975) or the like.

In the novel recombinant plasmid DNA thus obtained, there exists a vector DNA gene, e.g. β-lactamase (enzyme that destroys ampicillin) gene, of Escherichia coli plasmid pBR322. Therefore, the transformed Escherichia coli shows resistance to ampicillin. The following technique is used to pick up a strain with a novel recombinant having a gene which shows complementarity to the human interferon messenger RNA among these ampicillin resistant strains.

First, [^{32}P] labelled DNA is synthesized with the RNA having interferon mRNA activity described above as a template and the DNA is hybridized with mRNA extracted, without induction by poly(I): poly(C) (therefore, interferon mRNA synthesis is not induced), from the human fibroblasts

by incubating at a high temperature (e.g. 65°C) in a reaction
mixture containing, for example NaCl (e.g. 0.5 M). Then,
the hybridized DNA (Probe A) and non-hybridized DNA (Probe
B) are separated by hydroxyapatite column chromatography.

5   Next, filter-fixed DNAs of transformants are hybridized
separately with Probe B or Probe A according to the technique
of Grunstein-Hogness (Proc. Nat. Acad. Sci. USA, vol. 72,
p. 3961-3965, 1975) and strains having a DNA hybridizable
with Probe B but not or barely with Probe A are discerned

10   by autoradiography.

Then, plasmid DNA is isolated from each of the
discriminated strains and hybridized with mRNA having inter-
feron mRNA activity by incubating at a high temperature
(e.g. 53°C) in the presence of 80% (w/v) formamide, 0.4 M

15   NaCl, etc. Since the mRNA hybridized with cDNA portion of
the plasmid DNA from the above-described strain can be
retained on a nitrocellulose filter, whereas unhybridized
mRNA can not under certain conditions (refer to Example
below and Nygaard, A.P. & Hall, B. D., Biochem. Biophys.

20   Res. Commun. Vol. 12, p. 98-104, 1963) this mRNA can be
recovered selectively from the filter at a high temperature
(e.g. 60°C) in a solution such as 90% (v/v) formamide and
thereafter injected into oocytes of Xenopus laevis.

When interferon is synthesized in the oocytes,

25   the DNA used for hybridization must contain a DNA which is
complementary to interferon mRNA; and by this method, a
recombinant plasmid DNA having a gene showing complementarity
to the human fibroblast interferon mRNA can be isolated.

The recombinant plasmid DNA obtained above or

30   segments cleaved with a restriction endonuclease are labelled
with a radio isotope such as ^{32}P by the Nick translation method
(Rigby, et al., J. Mol. Biol. vol. 113, p. 237-251, 1977),
or the like, and used as a probe to obtain Escherichia coli
strains containing a recombinant plasmid having the inter-

35   feron mRNA sequence from the above ampicillin resistant
strains in the same way as described above. Several strains
thus obtained are cultured and the plasmid DNA is isolated
therefrom. The plasmid DNA is cleaved with a restriction

endonuclease to obtain the inserted DNA. The length of the inserted DNA is investigated to obtain a plasmid having an inserted DNA coding the entire region of the interferon protein. Primary structure of the inserted DNA of one of recombinant plasmids isolated by the above method is determined according to the Maxam-Gilbert method (Proc. Nat. Acad. Sci. U.S.A. vol. 74, p. 560-564, 1977) and is illustrated in the following Example. It has thus been shown that the recombinant plasmid of the invention contains the entire coding region of the human fibroblast interferon mRNA.

As outlined above, a DNA which codes for human fibroblast interferon polypeptide, especially a DNA which encompasses the entire coding region of the human fibroblast interferon mRNA, a recombinant plasmid containing the DNA and a microorganism containing the plasmid are prepared.

The base sequence of the DNA obtained above and the corresponding peptide sequence are illustrated in Table 5 below.

The base sequence in Table 5 is a preferred example for the expression of the DNA which codes for human interferon polypeptide. Since the amino acids in the peptide sequence in Table 5 may be coded for by a base triplet other than those in Table 5, base sequences of the DNA which codes for human interferon polypeptide other than that in Table 5 are also included in the present invention.

The determination of the base sequence of the DNA which codes for human interferon polypeptide according to the present invention has enabled the chemical synthesis of such DNA.

The present novel recombinant plasmids having a gene which encompasses at least the entire coding region of the human fibroblast interferon mRNA are very useful because they enable mass production of interferon in Escherichia coli or in eukaryotic cells which can be grown on a large scale.

Recombinant plasmids containing a DNA using, as a template, leucocyte mRNA or immune interferon mRNA can be prepared by the same method as mentioned above and such

plasmids are also expected to be useful for the mass produc-
tion of interferon.

One specific embodiment of the present invention
is illustrated by the following representative example.

Example

After priming of human fibroblasts by overnight
incubation with MEM culture medium (product of Nissui Seiyaku
Co., Ltd., Japan) containing human interferon which is pre-
pared according to the method described in Proc. Nat. Acad.
Sci. USA, 73, 520-523 (1976) (25 U/ml), the fibroblasts
were superinduced by adding 10 μg/ml of poly(I): poly(C)
(product of Galbiochem Co., USA) and 5 μg/ml of cycloheximide
to the medium. The priming and superinduction are carried
out according to the methods described in Brit. J. Exp. Path.,
39, 452-458 (1958) and Antimicrob. Agents Chemother., 2,
476-484 (1972), respectively.

After 4 hours, 1.5×10^9 superinduced human fibro-
blasts were destroyed by Teflon homogenizer (sold by Takashima
Shoten Co., Japan) at a temperature of 0 to 4°C in the
presence of 0.3% NP-40 (product of Daiichi Kagaku Co., Japan)
and 50 μg/ml heparin in RSB buffer (10 mM Tris-HCl, pH 7.5;
10 mM NaCl; 1.5 mM $MgCl_2$). Nuclei were removed by centrifu-
gation at 3000 rpm and 4°C for 10 minutes and 9.6 mg of
cytoplasmic RNA was obtained by extraction 3 times with
phenol.

The cytoplasmic RNA was precipitated with 67%
ethanol in the presence of 0.1M NaCl, dissolved in 10 ml of
1 mM EDTA solution and incubated at 65°C for 2 minutes.
Then, 2.5 ml of a salt solution at a high concentration
(0.5 M Tris-HCl, pH 7.5; 1 M NaCl; 50 mM EDTA) was added to
the above solution and the mixture was put on a column packed
with 0.15 g of an oligo(dT) cellulose (product of P-L
Biochemicals Co., USA) to adsorb mRNA containing poly(A).
Elution was then carried out with a salt solution at a low
concentration (10 mM Tris-HCl, pH 7.5) and water to isolate
250 μg of mRNA containing poly(A).

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The mRNA was precipitated with 67% ethanol in the presence of 0.1M NaCl and dissolved in 0.5 ml of 1 mM EDTA solution. The solution was incubated at 65°C for 2 minutes, subjected to centrifugation through a 5 - 25% sucrose-density gradient containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl and 1 mM EDTA (rotated at 35,000 rpm using the SW40 rotor of Beckmann Co., U.S.A.) at 4°C for 16 hrs. and fractionated into 20 fractions.

The interferon mRNA activity of each of these fractions was determined as mentioned above, and the results are shown in Table 1 below.

Table 1

Fraction No.	Interferon Activity
9	< 50 units/ml
10	44
11	550
12	52

The mRNA in Fraction No. 11 was approximately 5 µg. This mRNA and a reverse transcriptase were incubated at 37°C for an hour in 20 µl of a reaction mixture consisting of 5 µg mRNA; 0.5 mM dATP; 0.5 mM dTTP; 0.5 mM dGTP; 0.5 mM dCTP; 1 µg oligo(dT) (product of P-L Biochemicals Co., USA); 8 units reverse transcriptase (derived from Avian myeloblastisis virus, for example, product of Life Science Inc. Florida, USA); 5 mM $MgCl_2$; 30 mM NaCl; 5 mM mercaptoethanol; and 40 mM Tris-HCl (pH 8.0) and then deproteinized with phenol. After RNA was removed by treatment with 0.3 N NaOH at 37°C for 15 hours, the synthesized single stranded DNA was incubated at 37°C in 20 µl of a reaction mixture [the same mixture as described above except that mRNA and oligo(dT) were omitted] for one hour to synthesize 1.5 µg of a double stranded DNA.

The double stranded DNA was treated with Nuclease S_1 (product of Bethesda Research Laboratories Inc., USA which is referred to as BRL, hereinafter) in 50 µl of a reaction mixture (1.5 µg double stranded DNA: 1 mM $ZnCl_2$;

5 0.1 M sodium acetate, pH 4.5; 0.2 M NaCl; 0.05 unit S_1) at 37°C for 30 minutes and the enzyme was removed by phenol extraction. The DNA was precipitated with ethanol and then treated with a terminal transferase in 20 µl of a reaction mixture consisting of 1.5 µg DNA; 0.14 M potassium cacodylate,

10 pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM $CoCl_2$; 1 mM dATP; and 1 unit terminal transferase (product of BRL) at 37°C for 20 minutes to obtain about 1.5 µg of a product wherein 100 deoxyadenosine chains were formed at both 3' ends of the double-stranded DNA.

15 In an alternative procedure, 10 µg of Escherichia coli plasmid pBR322 DNA (product of BRL) was treated at 37°C for 2 hours with a restriction endonuclease EcoRI in 100 µl of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$; 0.1 M NaCl; 6 mM mercaptoethanol; and 10 units

20 EcoRI (product of BRL) leading to cleavage at the only cutting site in pBR322 DNA. The cut plasmid DNA was treated with an exonuclease derived from phage λ in 200 µl of a reaction mixture consisting of 10 µg DNA; 0.1 M Na-glycine, pH 9.5; 5 mM $MgCl_2$; 50 µg/ml albumin (product of

25 Merck & Co., USA); and 17.5 units λ exonuclease (product of Miles Laboratories Inc., USA) at 0°C for 90 minutes and the enzyme was removed by phenol extraction. The DNA was treated with a terminal transferase in 50 µl of a reaction mixture [10 µg DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris

30 (base); 0.1 mM dithiothreitol; 1 mM $CoCl_2$; 1 mM dTTP; 2 units terminal transferase] at 37°C for 20 minutes to obtain about 0.5 µg of a product wherein 100 deoxythymidine chains were formed at both 3' ends of plasmid pBR322 DNA described above.

35 Then, 0.02 µg of the synthesized double stranded DNA obtained above, and 0.1 µg of the plasmid pBR322 DNA were incubated for hybridization in a solution containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA at 65°C

for 2 minutes, at 45°C for one hour, at 37°C for one hour
and at room temperature for one hour. Then, Escherichia
coli X1776 was transformed with the hybridized recombinant
following the method of Enea et al.

5   About 4,000 ampicillin-resistant strains were
isolated by this method. 3,600 resistant strains were chosen,
and the DNA of each strain was fixed on nitrocellulose
filters in duplicate (Grunstein-Hogness Method).

        In a further procedure, $[^{32}P]$ labelled single stranded
10 DNA was synthesized (about 0.44 μg, specific radioactivity
approx. 6 x 10^8 c.p.m./μg) by reverse transcriptase in the
same way as that for single stranded DNA mentioned above
(dCTP was labelled with ^{32}P) using the interferon mRNA
fraction (about 10 μg) which had been extracted and partially
15 purified as described above, as a template. The DNA was
hybridized in 50 μl of a reaction mixture (25 μg mRNA; 0.45
μg single stranded DNA labelled with ^{32}P; 0.5 M NaCl; 25 mM
Pipes buffer, pH 6.5) at 65°C for 40 hours with 25 μg of
mRNA extracted from human fibroblasts which had not been
20 induced by poly(I): poly(C). The latter mRNA was prepared
by the same method used to extract poly(I): poly(C)-induced
mRNA. The reaction mixture was put on a column packed with
0.2 g of a hydroxyapatite, and elution was first carried out
with 5 ml of 0.14 M phosphate buffer (pH 6.5) to elute the
25 single stranded DNA, and then with 5 ml of 0.4 M phosphate
buffer to elute the DNA hybridized with RNA. As a result,
the DNA (about 90% of the total) (Probe A) which hybridized
with the mRNA mentioned above, and the DNA (about 10% of the
total) (Probe B) which did not hybridize were isolated.

30   Each probe was then hybridized separately with the
above DNA fixed on the nitrocellulose filters according to
the Grunstein-Hogness method. Four strains were identified
which reacted mainly to Probe B but little to Probe A under
autoradiography.

35   Table 2 shows the extent of reaction of the DNAs
from the four strains to each probe as revealed by auto-
radiogram.

Table 2

Ampicillin-resistant strains	Extent of Reaction of Probe with DNA in the strains	
	Probe A	Probe B
# 319	+ +	+ + + +
# 644	+	+ + +
# 746	-	+ +
#3578	+	+ + + + +

Plasmid DNA was isolated from cells of the four strains by the method of Currier and Nester (Analyt. Biochem. vol. 76, p. 431-441, 1976). Then, these DNAs were hybridized with the interferon mRNA as follows.

First, 5 µg of plasmid DNA was linearized by incubating with restriction endonuclease Hind III,which can be obtained from Haemophilus influenzae Rd,in 50 µl of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$; 50 mM NaCl; 6 mM mercaptoethanol; and 5 units Hind III (product of BRL) at 37°C for 2 hours. After deproteinization by phenol extraction, the DNA was precipitated with ethanol and dissolved in 20 µl of 80% (w/v) formamide. The solution was denatured at 85°C for 10 minutes and was then incubated in a solution consisting of 2.5 µg mRNA, 20 µl 80% (w/v) formamide, 20 mM Pipes buffer (pH 6.5), 0.4 M NaCl and 5 mM EDTA, at 53°C. Four hours later the mixture was mixed with 0.4 ml of 3 x SSC (1 x SSC corresponds to 0.15 M NaCl, 0.015 M sodium citrate) at 0°C, and was filtered through a nitrocellulose filter (diameter : 1 cm, pore size : 0.45 µm) at a rate of about 0.5 ml per minute. After washing the filter with about 1.5 ml of 2 x SSC, the filter was immersed in a solution consisting of 0.6 ml of 90% (v/v) formamide, 20 mM Pipes buffer, 0.1% SDS (sodium dodecylsulfate) and 5 mM EDTA. Incubation of the filter at 60°C for 2 minutes and the removal of the solution were repeated 3 times and the RNA eluted from the nitrocellulose filter into the solution (1.8 ml) was precipitated with ethanol in the presence of

0.1 M NaCl. The mRNA containing poly(A) was isolated from
the RNA by using oligo(dT) cellulose column chromatography,
dissolved in a mixture of 3 µl of 10 mM Tris-HCl (pH 7.5)
and 88 mM NaCl and injected into the oocytes of Xenopus

5   laevis. After 15 hours the interferon synthesized in the
oocytes was determined (antiviral activity).

Table 3 shows the interferon mRNA activity of the
mRNA which has hybridized with the DNA derived from the four
bacterial strains mentioned above.

10

Table 3

Bacterial strain	Interferon mRNA activity (unit/ml)
# 319	360
# 644	< 10
# 746	15
#3578	< 10
pBR322DNA	< 10

20

Five µg of plasmid DNA obtained from strain #319
by the Currier and Nester method was cleaved with restriction
endonuclease Hind III in the same manner as mentioned above.

25   The DNA and the recombinant plasmid βGpBR322 DNA (the vector
was pBR322) (obtained from the Institute for Molecular
Biology I of University of Zurich or prepared by the method
described in Nature 281, 40-46, 1979) containing rabbit
β-globin gene, separately or as a mixture, were hybridized

30   with a mixture of rabbit globin mRNA (obtained from rabbit
red blood cells) (1 µg) and interferon mRNA (2.5 µg) obtained
from human fibroblasts under the same conditions as mentioned
above. The mRNA which formed a hybrid was injected into the
oocytes of Xenopus laevis. The oocytes were then incubated

35   for 15 hours in Barth's culture medium (J. Embryol. Exp.
Morph. 7, 210, 1959) containing [^{3}H] labelled histidine,and
[^{3}H] labelled globin was isolated by acrylamide gel electro-
phoresis and determined quantitatively by fluorography

according to the method described in Eur. J. Biochem. <u>46</u>,
83-88, (1974). The interferon was determined by antiviral
activity as described above. The synthesis of rabbit
β-globin and the human interferon was determined in this way.
The result is shown in Table 4 below.

Table 4

D N A	Synthesized interferon activity	Amount of globin synthesized
# 319	200 (units/ml)	-
βGpBR322	35	+ + + +
mixture of both plasmids	160	+ + +

From the results of this experiment it has been
established that DNA of #319 has DNA (the interferon gene)
which forms a hybrid specifically with the interferon mRNA.

The DNA of #319 was cleaved with several restriction
endonucleases and a restriction endonuclease map, Fig. 1(a),
was made by agalose electrophoresis.

Restriction endonucleases, Pst I, Bgl II and Hind
III (sold by BRL, etc.) cleave #319 DNA at the sites illus-
trated in Fig. 1 (a).

The segments obtained by cleaving #319 DNA with
restriction endonucleases Pst I and Bgl II were isolated
and purified by gel electrophoresis according to the method
of Tabak & Flavell (Nucleic Acids Research, vol. 5, p. 2321-
2332, 1978). The segments were labelled with ^{32}P according
to the method of Rigby, et al. (J. Mol. Biol. vol. 113,
p. 237-251, 1977) and the labelled segment was used as a
probe. Several strains containing a plasmid which shows
complementarity to the probe were isolated from the above
ampicillin-resistant strains according to the above method
of Grunstein & Hogness (Proc. Nat. Acad. Sci. U.S.A., vol.
72, p. 3961-3965, 1975), namely the colony hybridization method.
Plasmid DNAs were obtained from each of the strains according

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to the above method of Currier-Nester and the inserted
portions thereof were cleaved with a restriction endonuclease
such as Hind III. The cut plasmid DNA segments were compared
in length and the longest plasmid DNA segment was selected.

5   The plasmid was named #319-13.

The restriction endonuclease map of the plasmid
is illustrated in Fig. 1 (b), which substantiates that the
novel plasmid has an mRNA sequence containing the mRNA
sequence of #319. Primary structure (base sequence) of the

10 mRNA sequence inserted in the plasmid of #319-13 was deter-
mined by the method of Maxam-Gilbert (Proc. Nat. Acad. Sci,
U.S.A. vol. 74, p. 560-564, 1977). The primary structure is
given in Table 5 below.

15

Table 5

```
                 -20                        -10                            1
        Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser Thr Thr Ala Leu Ser Met Ser Tyr
 5      GTC AAC ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC
        CAG TTG TAC TGG TTG TTC ACA GAG GAG GTT TAA CGA GAG GAC AAC ACG AAG AGG TGA TGT CGA GAA AGG TAC TCG ATG
                       20                        40                          60

                                 10
        Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu
        AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA
        TTG AAC GAA CCT AAG GAT GTT TCT TCG TCG TTA AAA GTC ACA GTC TTC GAG GAC ACC GTT AAC TTA CCC TCC GAA CTT
            80                 100                        120                        140

            30                        40                        50
10      Tyr Cys Leu Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln Gln Phe Gln Lys Glu Asp Ala
        TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC
        ATA ACG GAG TTC CTG TCC TAC TTG AAA CTG TAG GGA CTC CTC TAA TTC GTC GAC GTC GTC AAG GTC TTC CTC CTG CGG
              160                    180                        200                        220

                             60                        70                        80
        Ala Leu Thr Ile Tyr Glu Met Leu Gln Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glu
        GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT GAG
        CGT AAC TGG TAG ATA CTC TAC GAG GTC TTG TAG AAA CGA TAA AAG TCT GTT CTA AGT AGA TCG TGA CCG ACC TTA CTC
15             240                       260                        280                    300

                                 90                        100
        Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu
        ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA CTG GAG
        TGA TAA CAA CTC TTG GAG GAC CGA TTA CAG ATA GTA GTC TAT TTG GTA GAC TTC TGT CAG GAC CTT CTT TTT GAC CTC
                  320                   340                       360                        380

              110                       120                        130
20      Lys Glu Asp Phe Thr Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu
        AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG
        TTT CTT CTA AAG TGG TCC CCT TTT GAG TAC TCG TCA GAC GTG GAC TTT TCT ATA ATA CCC TCC TAA GAC GTA ATG GAC
                     400                   420                        440                        460

                             140                       150
        Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg
        AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT TAC TTC ATT AAC AGA
        TTC CGG TTC CTC ATG TCA GTG ACA CGG ACC TGG TAT CAG TCT CAC CTT TAG GAT TCC TTG AAA ATG AAG TAA TTG TCT
25                 480                   500                        520                        540

        160         166
        Leu Thr Gly Tyr Leu Arg Asn
        CTT ACA GGT TAC CTC CGA AAC TGA AGA TCT CCT AGC CTG TGC CTC TGG GAC TGG ACA ATT GCT TCA AGC ATT CTT CAA
        GAA TGT CCA ATG GAG GCT TTG ACT TCT AGA GGA TCG GAC ACG GAG ACC CTG ACC TGT TAA CGA AGT TCG TAA GAA GTT
                      560                  580                        600                        620

        CCA GCA GAT GCT GTT TAA GTG ACT GAT GGC TAA TGT ACT GCA TAT GAA AGG ACA CTA GAA GAT TTT GAA ATT TTT ATT
30      GGT CGT CTA CGA CAA ATT CAC TGA CTA CCG ATT ACA TGA CGT ATA CTT TCC AGT GAT CTT CTA AAA CTT AAA AAA TAA
                      640                  660                        680                        700

        AAA TTA TGA GTT ATT TTT ATT TAT TTA AAT TTT ATT TTG GAA AAT AAA TTA TTT TTG GTG CAA AAG TCA AAA AAA
        TTT AAT ACT CAA TAA AAA TAA ATA AAT TTA AAA TAA AAC CTT TTA TTT AAT AAA AAC CAC GTT TTC AGT TTT TTT
                      720                  740                        760
```

35

The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences.

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It is important that in the sequence there exist without any errors the base sequence (three base pairs) corresponding to the amino acid sequence from the amino-terminal to 13th amino acid of the human fibroblast interferon

5 reported by Knight, et al. (Science vol. 207, p. 525-526, 1980). This fact establishes that the #319-13 plasmid of the present invention has the human fibroblast interferon mRNA sequence.

Further, it is apparent from the data of the primary

10 sequence that the plasmid encompasses the entire coding region of the protein of the above mRNA and probably the coding region of the signal peptide.

Therefore, transformation of the plasmid or mRNA inserted therein to other expression plasmids enables a host

15 such as Escherichia coli to produce interferon. For such purposes, the #319-13 plasmid which is named TpIF 319-13, transformed in Escherichia coli X1776, has been deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number ATCC 31712 and is

20 freely available to the public.

What is Claimed is

1.   A DNA which codes for a polypeptide with interferon activity.

2..   A cloned DNA showing complementarity to human interferon messenger RNA.

3.   The cloned DNA according to claim 2, wherein the messenger RNA is human fibroblast interferon messenger RNA.

4.   A cloned DNA which codes for human interferon polypeptide.

5.   The cloned DNA according to claim 4, wherein the polypeptide is human fibroblast interferon polypeptide.

6.   A recombinant plasmid wherein a DNA showing complementarity to human interferon messenger RNA is inserted in a vector DNA.

7.   The recombinant plasmid according to claim 6, wherein the messenger RNA is human fibroblast interferon messenger RNA.

8.   The recombinant plasmid according to claims 6 or 7, wherein the plasmid is an Escherichia coli plasmid.

9.   The recombinant plasmid according to claim 8, wherein the plasmid is selected from pBR322, pCR1, pMB9 and pSC1.

10.   The recombinant plasmid TpIF 319-13.

11.   A microorganism containing the recombinant plasmid defined in claim 6.

12.   A microorganism containing the recombinant plasmid defined in claim 7.

13.   The microorganism according to claim 11 which is Escherichia coli X1776.

14. The microorganism according to claim 12 which is Escherichia coli χ1776.

15. Escherichia coli χ1776/TpIF 319-13 ATCC 31712.

16. A process for producing a DNA which codes for a polypeptide with interferon activity by recombinant DNA technology.

17. The process according to claim 16, wherein the polypeptide is the human fibroblast interferon polypeptide.

18. A process for producing a DNA which codes for a polypeptide with interferon activity by using human interferon messenger RNA as a template.

19. The process according to claim 18, wherein the DNA is a cloned DNA showing complementarity to human interferon messenger RNA.

20. The process according to claim 19, wherein the messenger RNA is human fibroblast interferon messenger RNA.

21. A process for producing a recombinant plasmid, which comprises inserting a DNA showing complementarity to human interferon messenger RNA in a vector DNA.

22. The process according to claim 21, wherein the messenger RNA is human fibroblast interferon messenger RNA.

23. The process according to claim 21 or 22, wherein the vector DNA is an Escherichia coli plasmid.

24. The process according to claim 23, wherein the plasmid is selected from pBR322, pCRl, pMB9 and pSCl.

25. The process according to claim 21, wherein the recombinant plasmid is TpIF 319-13.

26. A process for producing a microorganism containing a recombinant plasmid defined in claim 21 or 22, which comprises transforming a microorganism with said
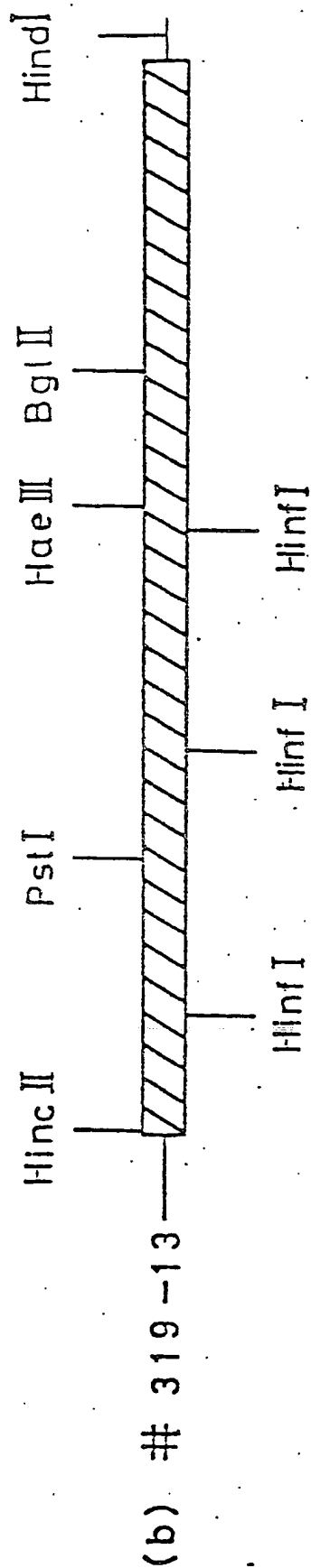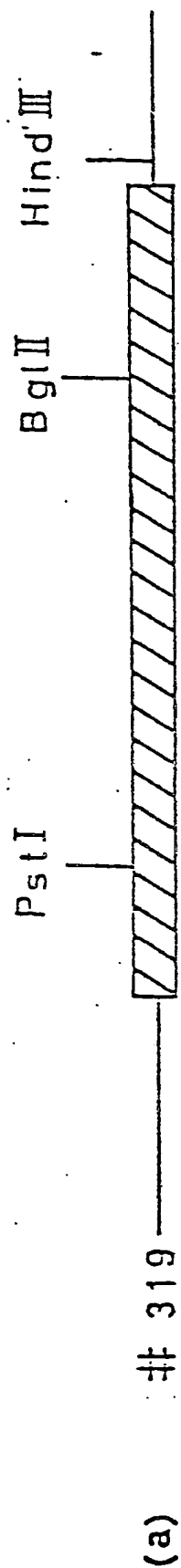
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recombinant plasmid in a conventional manner.

27. The process according to claim 26, wherein the microorganism is *Escherichia coli* χ1776.

28. The process according to claim 26, wherein the recombinant plasmid is TpIF 319-13.

29. The process according to claim 26, wherein the microorganism containing a recombinant plasmid is *Escherichia coli* χ1776 TpIF 319-13 ATCC 31712.

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Fig. 1

(a) ＃319

PstI   BglⅡ   Hind'Ⅲ

(b) ＃319-13

HincⅡ   PstI   HaeⅢ BglⅡ   HindI

HinfI   HinfI   HinfI